Expression of fatty acid-CoA ligase 4 during development and in brain

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Abstract Fatty acid utilization is initiated by fatty acid-CoA ligase, which converts free fatty acids into fatty acyl-CoA esters. We have cloned previously the human long-chain fatty acid-CoA ligase 4 (FACL4), which is a central enzyme in controlling the free arachidonic acid level in cells and thereby regulating eicosanoid production. We report here the expression of this gene in tissues, particularly in different parts of the brain. We found that FACL4 encoded a 75 kDa enzyme and that there was a modified translation product expressed in the brain. FACL4 was expressed in early stages of development with a significant amount of FACL4 mRNA detected in an E7 mouse embryo. In addition, FACL4 was highly expressed in both adult and newborn mouse brain especially in the granule cells of the dentate gyrus and the pyramidal cell layer of CA1 in hippocampus, and the granular cell layer and Purkinje cells of the cerebellum.

Key words: Arachidonic acid; Fatty acid-CoA ligase; Gene expression; Mouse embryo; Hippocampus

1. Introduction

Arachidonic acid (AA; 20:4, n-6), an essential polyunsaturated fatty acid, is a substrate for an important class of lipid mediators, eicosanoids, including prostaglandins, thromboxanes and leukotrienes [1]. Free AA in cells is normally maintained at a low level, so constitutive eicosanoid synthesis is limited under unstimulated conditions. AA entering cells exogenously or released endogenously is rapidly converted to AA-CoA esters by the catalytic action of fatty acid-CoA ligase (FACL), particularly by the AA-preferring FACL4 [2,3]. FACL is a key enzyme in controlling the free fatty acid pools and is essential for usage of free fatty acids as building blocks and energy sources. To date, five isoforms of this gene have been cloned in mammals with diverse substrate specificity and tissue distribution [3,4]. We have reported previously the cloning and tissue expression pattern of the isoform 4 of FACL in humans [3]. Compared to other isoforms, isoform 4 of both human and rat highly prefers AA and eicosapentanoic acid (EPA) as substrates [3,5]. Therefore, FACL4 plays an important role in AA metabolism. Our previous analysis by Northern blotting indicated that FACL4 is highly expressed in brain, placenta, testis, ovary, spleen and adrenal cortex. Interestingly, we noticed that a different size of FACL4 mRNA was present in brain compared to that in other tissues, which suggested a brain-specific alternative transcript [3]. To elucidate more on tissue-specific and developmental regulation of FACL4 expression, we further characterized the protein expression of FACL4 in the brain and other tissues, the mRNA and protein expression pattern in brain structure of mice, and mRNA expression during development.

FACL4 was found to be deleted in a family with Alport syndrome, elliptocytosis, and mental retardation [6]. This raised the possibility that deficiency of FACL4 contributes to the development of mental retardation. Therefore, determination of expression pattern of FACL4 in brain structure and during development will be fundamental in understanding the potential role of FACL4 in brain function.

2. Materials and methods

2.1. Immunoblotting

The experiment was performed as described previously [7]. A polyclonal anti-FACL4 antibody was raised in rabbit against a synthesized peptide 'H_2N-MAKRIKAKPTSDKPGC-amide' which corresponds to the deduced N-terminal sequence of human FACL4. The anti-FACL4 antibody was then affinity-purified and used as the primary antibody (1:3000 dilution). In peptide competition, the FACL4 N-terminal peptide (0.1 mg/ml) was incubated with the anti-FACL4 antibody at a 10:1 (v/v) ratio overnight at 4°C before the antibody was applied to the blot. This anti-FACL4 antibody subsequently was found to cross-react with mouse FACL4 in both Western blotting and immunostaining.

2.2. Northern blotting

This analysis was carried out as described previously [3]. Digoxigenin-labeled RNA probes were prepared by in vitro transcription using the DIG RNA-labeling kit (Boehringer Mannheim). The mouse FACL4 cDNA construct pSL147 was linearized by *Eco*RI or *Eco*RV digestion, and an anti-sense RNA probe of 682 bp was generated under the catalytic action of T3 RNA polymerase. The complementary sense probe was produced by T7 RNA polymerase, and used as a control for in situ hybridization.

2.3. Immunohistochemical staining

Paraffin-embedded mouse embryo sections were purchased from Novagen. The sections were deparaffinized and the endogenous peroxidase was eliminated by incubating in 3% hydrogen peroxide for 5 min. Adult and newborn mouse brains were embedded in OCT medium (VWR) and 6 μM thick sections were picked up on Superfrost Plus slides (Fisher Scientific). The sections then were fixed in cold acetone for 10 min. The endogenous peroxidase in the tissues was eliminated by incubating in 0.1% hydrogen peroxide for 15 min. The sections were blocked by incubating with 5% normal goat serum, 1% bovine serum albumin for 30 min, and then the anti-FACL4 antibody was diluted 1:200, applied to the slides and incubated overnight. A secondary antibody (biotin-conjugated goat anti-rabbit, Jackson Immunoresearch) was applied at a 1:1000 dilution and incubated for 30 min. Positive control was performed using an S-100 antibody (Sigma), and negative controls consisted of omitting the anti-FACL4 antibody, and/or secondary antibody, or pre-incubating the antibody with the immunizing peptide overnight. ABC reagent was prepared according to the manufacturer's instructions (Vector Laboratories)

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and applied to the sections for 30 min. The signal was detected using a 3,3-diaminobenzidine tetrahydrochloride (DAB) kit (Vector Laboratories), followed by counterstaining with Gill's hematoxylin, dehydrating, and mounting with Cytoseal 60 medium (VWR).

2.4. Tissue in situ hybridization

Both anti-sense and sense (negative control) RNA probes were prepared as described above, and diluted to 1 μg/ml in hybridization buffer (50% formamide, 2×SSC, 10% dextran sulfate, 0.01% herring sperm DNA, 0.02% SDS). After dehydration of the slides, 20 μl of the probe solution was applied to each slide. The slides were heated to 95°C for 4 min followed by hybridization at 55°C for 4 h.

Post-hybridization washes consisted of 2×SSC overnight, three times in 50% formamide/1×SSC for 20 min at 55°C, twice in 1×SSC for 15 min, and a final rinse in 1×TBS. The sections were blocked in 5% normal sheep serum, 0.03% Triton X-100 in Buffer 1 (100 mM Tris–HCl, pH 7.5, 150 mM NaCl) for 15 min, and then 200 μl of detection antibody (alkaline phosphatase-conjugated mouse anti-biotin, Jackson Immunoresearch) was applied for 1 h. The slides were washed three times in Buffer 1 for 10 min, and then equilibrated in Buffer 3 (100 mM Tris–HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) for 5 min. They subsequently were immersed in substrate solution (4.5 μl/ml NBT, 3.5 μl/ml BCIP in Buffer 3) and allowed to develop for 60 min. The reaction was terminated in Buffer 4 (10 mM Tris–HCl, pH 8.0, 1 mM EDTA), and the slides were counterstained with Nuclear Fast Red, and mounted with Cytoseal 60 (VWR).

3. Results

3.1. FACL4 protein has a different electrophoretic mobility in the brain and is not detectable in the liver

Our previous results by Northern analysis revealed that FACL4 mRNA has a different size in the brain compared to other tissues, and that little mRNA was detected in the liver. To determine the protein expression in different tissues, we examined the expression of FACL4 protein in the human heart, brain and liver by Western blotting (Fig. 1). One specific band corresponding to 75 kDa was detected in the heart, and mobility of the band was the same as in the control, which was HEK293 epithelial cells transiently transfected with a human FACL4 cDNA. There also was a band of apparently higher molecular weight in control cells and the brain lysates which also could be competed away with the FACL4 peptide. Whether this is a precursor of FACL4 or another

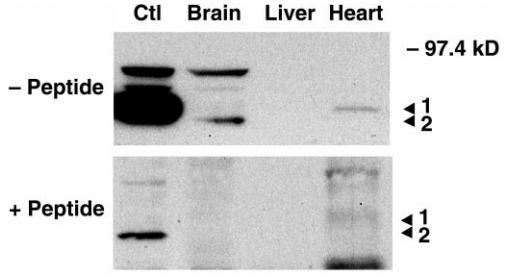

Fig. 1. Expression of FACL4 protein in human tissues. One hundred μg of whole protein lysate from various tissues (Clontech), and 50 μg of lysate from 293 cells transiently transfected with a human FACL4 cDNA construct (control) were separated by SDS–PAGE. The blot was probed with the anti-FACL4 antibody which was pre-incubated with or without the N-terminal peptide of FACL4. The binding was specific as it was blocked by pre-incubation with the FACL4 N-terminal peptide. − Peptide, without peptide competition; + Peptide, with peptide competition. Arrowhead 1 points to the position of FACL4 bands in the cell lysate and the heart, and arrowhead 2 indicates that in the brain.

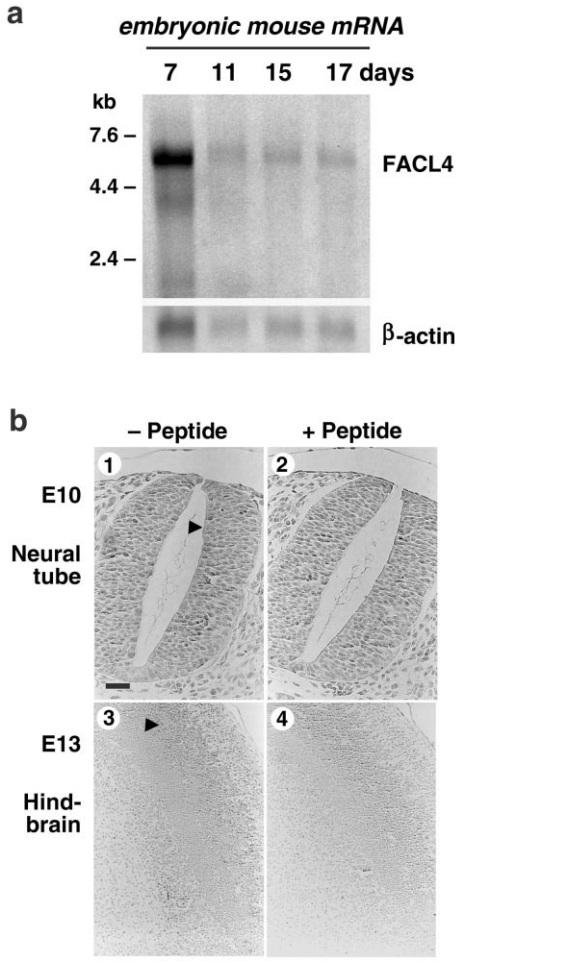

Fig. 2. Expression of FACL4 during early development. (a) Northern blot analysis of the expression of FACL4 during mouse embryonic development. One μg of poly(A)$^+$ RNA from mouse embryos of 7, 11, 15 and 17 days old, respectively was separated and then immobilized to the membrane (Clontech). Northern blot analysis was carried out by hybridizing to a digoxigenin-labeled probe containing the anti-sense mouse FACL4 RNA. The blot was also probed with a β-actin anti-sense RNA for loading normalization. The number to the left hand side indicates the position of the size marker. (b) Immunohistochemical staining of the E10 and E13 mouse embryonic sections (40×). − Peptide, without peptide competition (panels 1 and 3); + Peptide, with peptide competition (panels 2 and 4). The arrowhead in panel 1 indicates the positive signals in the neural tube. The arrowhead in panel 3 refers to the staining in the hindbrain. Scale bar = 80 μm.

isoform of FACL that cross-reacts with the FACL4 antibody is not clear. In contrast to the heart, with the same amount of whole protein lysate, no FACL4 protein was detected in the liver, which agrees with the previous finding that there is an extremely low mRNA level in the liver [3]. These two findings suggest that a low rate of transcription of the FACL4 gene is the basis for the low level of protein in the liver. In addition, we observed a FACL4 band with a slightly faster mobility in the brain (Fig. 1). Densitometry analysis indicated that the FACL4 band was 5.5-fold more intensive in the brain than that in the heart, indicating that FACL4 protein is expressed at a much higher level in the brain than in the heart.

3.2. FACL4 is expressed during early development

Previously, we have observed that human FACL4 is ex-

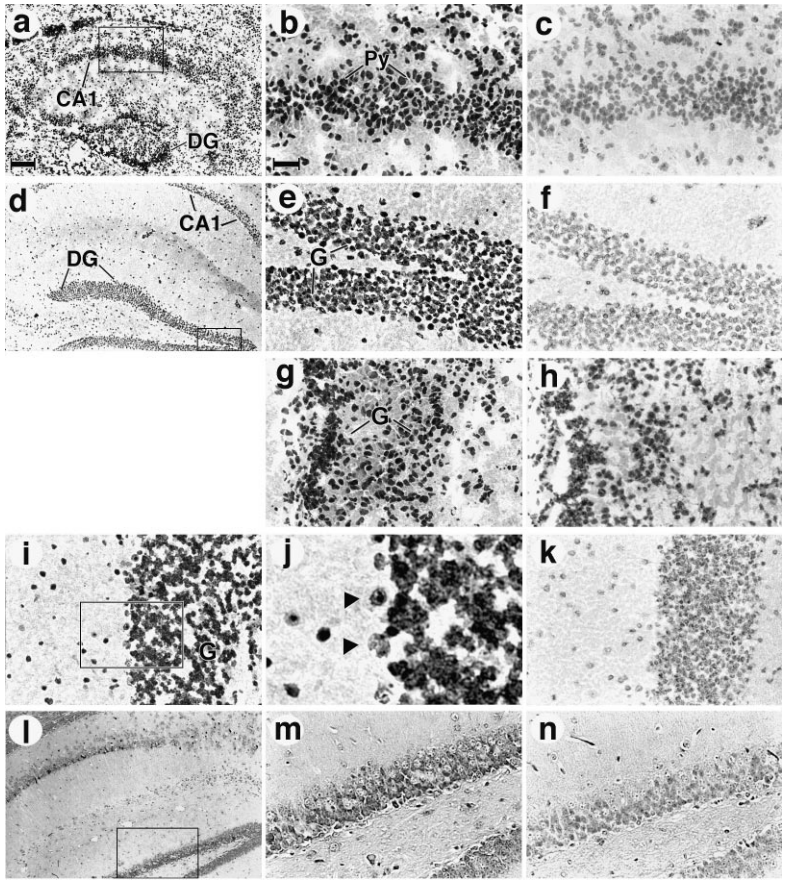

Fig. 3. Expression of FACL4 in mouse hippocampus and cerebellum. a through k are immunohistochemical staining with the anti-FACL4 antibody. a, b, c are hippocampal coronal sections of newborn mice (< 24 h postpartum), and d, e, f are sagittal sections from the hippocampus of adult mice. b and e show the indicated cropped regions in a and d under higher magnification. g and h are coronal sections of newborn cerebellum, and i and k are sagittal sections of adult cerebellum. j is the enlarged view of the cropped region in i, and the arrowheads indicate the positively stained Purkinje cells. In c, f, h and k, the antibody was pre-incubated with the FACL4 peptide (negative controls). l, m, n are in situ hybridization with the FACL4 anti-sense (l, m) and sense (n, control) RNA probes. m is an enlarged view of the cropped region in l under higher magnification. DG, dentate gyrus; Py, pyramidal cell layer; G, granule cells; CA1, region CA1. a through k were counterstained with hematoxylin. l through n were counterstained with Nuclear Fast Red. Magnification: a, d, l are 10×, and all the rest are 40×. Scale bar = 320 μm for panels a, d, l. Scale bar = 80 μm for panels b, c, e, f, g, h, i, k, m, n.

pressed widely in various adult tissues [3]. We next asked if FACL4 is expressed during early stages of development using Northern blot analysis. A mouse anti-sense RNA probe was prepared, and hybridized to poly(A)$^+$ RNAs from mouse embryos of 7, 11, 15 and 17 days old, respectively. A single band of 5.0 kb was detected in all samples (Fig. 2a). The expression levels of FACL4 in prenatal mice were similar except that in the E7 embryo which appeared even higher than in the older embryos. We noticed that the intensity of the internal control band, β-actin, was also high in the E7 embryo mRNA, so it is likely that the intensive band of FACL4 resulted from overloading. Next, we performed immunohistochemical staining with sections from E10 and E13 mouse embryos, and the results confirmed that FACL4 was expressed in embryos (Fig. 2b). Moreover, strong staining was detected in the central and peripheral nervous systems. Specifically, the lining of the neural tube was positively stained in the E10 embryo (Fig. 2b, panel 1), as was the hindbrain, and regions of the spinal cord in the E13 embryo (Fig. 2b, panel 3). We conclude that the FACL4 gene is expressed early during development, and that expression is detectable as early as the E7 embryo.

3.3. *FACL4 is highly expressed in the hippocampus and cerebellum*

In our pilot in situ hybridization with the whole mouse brain, the hippocampus and cerebellum appeared to have a strong expression of FACL4 mRNA (data not shown). To examine the distribution of FACL4 in the structure of developing and adult brains, we performed immunohistochemical staining and in situ hybridization on mouse brain sections. We found that hippocampal sections from either newborn or adult brain (Fig. 3a, b, d, e) stained positively with the anti-FACL4 antibody. The specific staining patterns (Fig. 3a, b, d, e versus peptide competition controls c, f) indicated that FACL4 is highly expressed in the hippocampus in both newborn and adult brains: we detected strong signals in the densely packed granule cells of the dentate gyrus (Fig. 3e) and also the pyramidal cell layer of region CA1 (Fig. 3b). In addition, the staining pattern in the cerebellum of newborn and adult mice was examined. Compared to the adult cerebellum (panel i), the structure of the newborn cerebellum is not fully established yet, and showed morphology with less characteristics (panels g, h). The dense granular layer of both newborn and adult showed a strong signal (Fig. 3g, i). The

Purkinje cell, which is the only output element of the cerebellar cortex and forms the sole link between the cerebellar cortex and the cerebellar nuclei, also stained positively (Fig. 3j). In parallel, serial sections of the section shown in Fig. 3d were hybridized to FACL4 RNA probes (Fig. 3l, m, n). A high level of expression of FACL4 was detected in the granule cells using the anti-sense RNA probe (panel m), which is consistent with the immunostaining pattern (panel e). In situ hybridization to the adult cerebellum also showed a similar expression pattern as revealed by the immunohistochemical staining (data not shown). Taken together, these results indicate that FACL4 is highly expressed in the hippocampus and cerebellum.

4. Discussion

The absence of the FACL4 gene in a family of non-syndromic X-linked mental retardation raised the possibility that deficiency of this gene could lead to Xq23-linked mental retardation. So far, five unrelated pedigrees with mental retardation have been characterized around Xq22~26 (MRX 23, 27, 30, 35, 47) [8–12], and only one of them (MRX 30) has been identified as a mutation in PAK3 (p21-activated kinase) [13]. Our immunohistochemical staining and in situ hybridization results showed that the expression pattern of FACL4 is very similar to PAK3 in developing and adult brain, suggesting that FACL4 could play an important role in normal brain functions, particularly in learning and memory. Therefore, deficiency of this gene might have serious clinical consequences.

Neuropsychological studies and neurophysiological experiments have revealed that the hippocampus plays a key role in certain aspects of learning and memory, and the cerebellum is essential to the execution of specific movements, i.e. motor coordination. The strong expression of FACL4 in both the hippocampus and the cerebellum suggests that the function of this enzyme may be essential to the normal function of these two structures. We speculate that this is the consequence of the structural and regulatory roles of the product of FACL4, arachidonoyl-CoA esters. In addition, this enzyme may also exert effects on normal brain function via controlling the free AA pool, as it has been shown that AA uptake and metabolism are dynamically regulated in brain, and AA acts directly on several aspects of brain function [14,15].

In a previous study, expression of other isoforms of FACL was detected at different levels in adult rat brains of varying ages [16]. Here we detected expression of the AA-preferring FACL4 in prenatal mice. Whether other isoforms are also expressed in the embryonic stages awaits investigation. The expression of FACL1 mRNA was at a constant level during postnatal brain development. In contrast, FACL2 mRNA increased gradually and reached a maximum in the adult brain, and FACL3, which is highly expressed in the brain, showed an increase in the early stages and a dramatic decrease in the late stages of postnatal development of rat adult brain [16]. We observed a higher expression of FACL4 mRNA in newborn than in adult mouse brain by in situ hybridization, which also suggests that this isoform may be developmentally regulated. The presence of multiple FACL isoforms in the brain and the differential regulation during development suggest that each isoform plays a distinct role in brain lipid metabolism. It is likely that each isoform is regulated spatially and temporally. Also, each isoform may localize to a subset of intracellular membranes, may prefer a specific fatty acid pool, then may drive the free fatty acids to different metabolic pathways. This hypothesis is supported by the observation that triacsin C exerted differential effects on lipid synthesis: it blocked synthesis of neutral lipids but not the incorporation of fatty acids into phospholipids [17]. One interpretation is that different FACL isoforms divert fatty acid into separate pathways of lipid synthesis, and these isoforms vary in their sensitivity to triacsin C.

Our previous Northern analysis suggests the presence of an alternative transcript in the brain. This transcript is most likely to result from transcription driven by a brain-specific promoter, and alternative splicing occurs between the first and second exons. In this study, we observed a FACL4 protein band with a different electrophoretic mobility in the brain which raised the possibility of tissue-specific alternative translation. The presence of heterogeneous amino termini has been reported in the FACL3 isoform, which is also highly expressed in the brain [18]. However since the anti-FACL4 antibody was raised against the most N-terminal sequence of FACL4 (see Section 2) and it could bind to both the brain and the heart forms of FACL4 (Fig. 1), it is unlikely that these two forms result from alternative translation. More likely, the different mobility is due to proteolysis, or post-translational modifications such as N-glycosylation. It has been shown previously that some membrane proteins are produced from precursors by furin-like proteinase. Amino acid sequence [3] analysis revealed that there is a basic tetrapeptide near the C-terminus of FACL4, from position 649 to 652 (KLKR) which is similar to a consensus sequence for proteolytic cleavage by furin-like proteinase [RX(K/R)R] [19]. In addition, there are two putative sites for N-glycosylation, located at amino acid residues 153 and 484 which match the consensus sequence, NXS/T [20]. Whether these potential sites are responsible for post-translational modification in a tissue-specific manner awaits further investigation.

There was no detectable mRNA expression of FACL4 in human liver [3] in contrast to the high expression in the hippocampus and cerebellum, especially during early stages of development. This suggests that FACL4 might not be essential for the synthesis of fuel components, which is a major function of the liver, whereas it might be central in AA recycling into phospholipids, particularly in the brain. The presence of an alternative transcript and a different FACL4 protein product in the brain strongly suggests that the expression and subcellular distribution of FACL4 could be regulated differentially in the brain compared to other tissues, and this type of regulation has been reported in previous examples [21,22]. The distribution of FACL4 in brain structure suggests that the function of the FACL4-derived arachidonoyl phospholipids may relate to intellectual capability and coordination skills.

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